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Fatty Acid Substitutions Modulate the Cytotoxicity of Puwainaphycins/Minutissamides Isolated from the Baltic Sea Cyanobacterium *Nodularia harveyana* UHCC-0300

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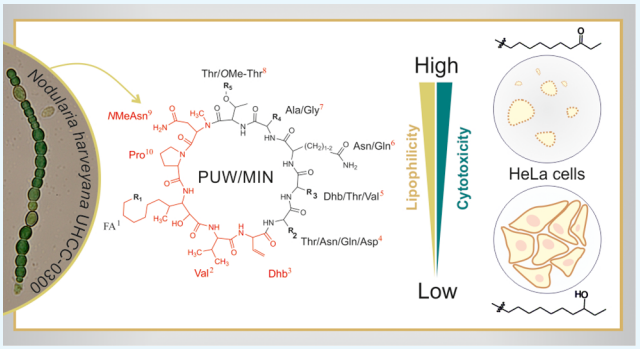
ABSTRACT: Puwainaphycins (PUW) and minutissamides (MIN) are structurally homologous cyclic lipopeptides that exhibit high structural variability and possess antifungal and cytotoxic activities. While only a minor variation can be found in the amino acid composition of the peptide cycle, the fatty acid (FA) moiety varies largely. The effect of FA functionalization on the bioactivity of PUW/MIN chemical variants is poorly understood. A rapid and selective liquid chromatography−mass spectrometry-based method led us to identify 13 PUW/MIN (**1**−**13**) chemical variants from the benthic cyanobacterium *Nodularia harveyana* strain UHCC-0300 from the Baltic Sea. Five new variants identified were designated as PUW H (**1**), PUW I (**2**), PUW J (**4**), PUW K (**10**), and PUW L (**13**) and varied slightly in the peptidic core composition, but a larger variation was observed in the oxo-, chloro-, and hydroxy-substitutions on the FA moiety. To address the effect of FA substitution on the cytotoxic effect, the major variants (**3** and **5**−**11**) together with four other PUW/MIN variants (**14**−**17**) previously isolated were included in the study. The data obtained showed that hydroxylation of the FA moiety abolishes the cytotoxicity or significantly reduces it when compared with the oxo-substituted C_{18}-FA (compounds **5**−**8**). The oxo-substitution had only a minor effect on the cytotoxicity of the compound when compared to variants bearing no substitution. The activity of PUW/MIN variants with chlorinated FA moieties varied depending on the position of the chlorine atom on the FA chain. This study also shows that variation in the amino acids distant from the FA moiety (position 4−8 of the peptide cycle) does not play an important role in determining the cytotoxicity of the compound. These findings confirmed that the lipophilicity of FA is essential to maintain the cytotoxicity of PUW/MIN lipopeptides. Further, a 63 kb puwainaphycin biosynthetic gene cluster from a draft genome of the *N. harveyana* strain UHCC-0300 was identified. This pathway encoded two specific lipoinitiation mechanisms as well as enzymes needed for the modification of the FA moiety. Examination on biosynthetic gene clusters and the structural variability of the produced PUW/MIN suggested different mechanisms of fatty-acyl-AMP ligase cooperation with accessory enzymes leading to a new set of PUW/MIN variants bearing differently substituted FA.



INTRODUCTION

Cyanobacteria are prolific producers of a large array of natural products, including peptides, polyketides, alkaloids, lipids, polyketones, and heterocyclic compounds.[1] They are also known to produce cyanotoxins during periodic blooms that have largely occurred in the last 20 years.[2−4] Among peptides, cyclic lipopeptides (CLPs) have received considerable attention for their wide range of bioactivities.[5−8] CLPs consist of a peptidic backbone with a diverse array of proteinogenic and non-proteinogenic amino acids attached to a fatty acid (FA) tail forming a cyclic ring structure.[9] The structure of CLPs substantially differs on amino acid composition and substitutions on the FA moiety. This unique structure and the resulting amphipathic molecular nature of CLPs promote integration into the membrane of the target organism, making them membrane-active compounds, such as surfactin, fengycin, iturins, and daptomycins.[10] CLPs are reported to possess significant antifungal[11] and cytotoxic activities[12−14] and also

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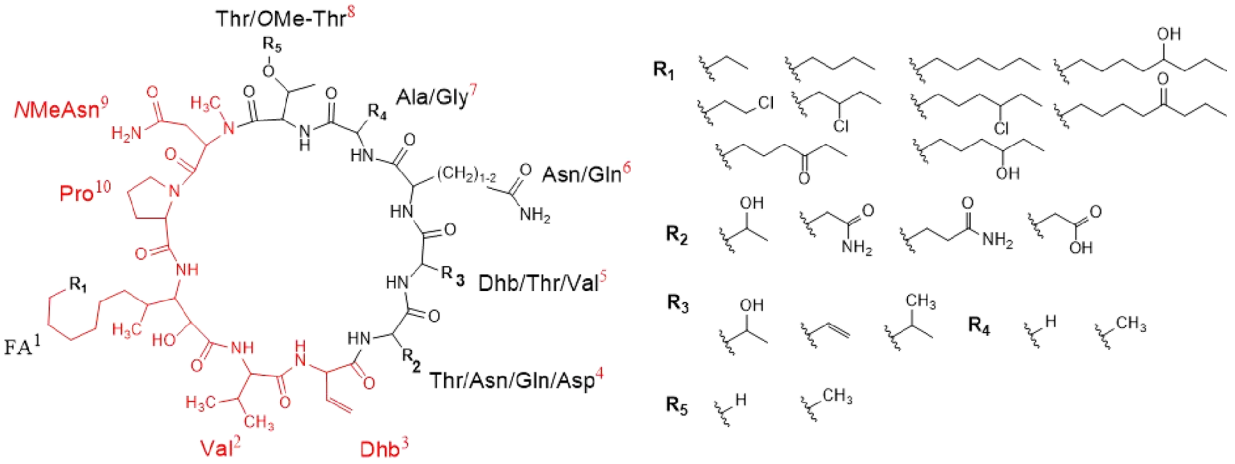

Figure 1. General structure of puwainaphycin and minutissamides. Conserved amino acids are depicted in red, and the variation among amino acid compositions is depicted in gray. FA[1] is the position for FA elongation, whereas R_1−R_5 are the variably functionalized alkyl substitutions. Dhb = dehydrobutyrine.

often used as biocontrol agents due to their antagonistic activity against a wide range of potential phytopathogens.[15−17]

The structure and the FA moiety of cyanobacterial CLPs differ substantially ranging from fully saturated FA chains, including puwainaphycins (PUW),[18] minutissamides (MIN),[19,20] muscotoxins,[21] and laxaphycins,[22] to polyunsaturated FAs such as in anabaenolysins.[12] PUW and MIN are structurally homologous amphipathic CLPs featuring a 10-membered peptide ring cyclized to form a lactam ring between an amino acid and an amino group bearing the FA moiety (*β*-amino FA) forming a lipid tail.[18−20,23] PUW/MIN has been reported from the genera *Cylindrospermum*, *Symplocastrum*, and *Anabaena*.[24] Twenty-one variants have been reported so far with the structural diversity arising from the differences in their peptide core as well as the FA substitution of different lengths (C_{10}−C_{18}) (Figure 1). A wide range of bioactivities have been reported for PUW/MIN variants, including cardiovascular activity, anti-proliferative activity, and antifungal activity.[11,18−20,25,26] PUW/MIN chemical variants are synthesized by a hybrid non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) pathway that is accompanied by a set of tailoring enzymes.[23] PUW/MIN biosynthesis begins with fatty-acyl-AMP ligases (FAAL) that are responsible for the activation of FAs, which are subsequently elongated by type I PKS modules encoded by PuwB and PuwE proteins.[24] The variability of the FA length is achieved by the broad substrate specificity of the PUW/MIN FAAL enzymes. However, some of the PUW/MIN biosynthetic gene clusters encode two alternative starter modules, each of which activates a different set of FAs.[24] The variability of the FA is further increased through substitution catalyzed by the PuwK halogenase, the PuwJ oxygenase, and the PuwL O-acetyltransferase.[24]

We report here the discovery of eight known (**3**, **5**−**9**, **11**, and **12**) and five new variants (**1**, **2**, **4**, **10**, and **13**) of PUW/MIN from the *Nodularia harveyana* strain UHCC-0300 using a previously developed rapid and selective mass spectrometry-based method for *β*-amino FA lipopeptide analysis[27] (Table 1). This method is based on high-performance liquid chromatography connected to tandem mass spectrometry with high-resolution mass spectrometry (HPLC−HRMS/MS) and allows the sensitive and efficient detection of PUW/MIN. The variability observed among the five new variants was mainly due to the difference in their amino acid composition. Finally, we study and report the effects of length and substitution (oxo-, hydroxy-, and chloro-) on the FA moiety as well as the amino acid position toward the change in the cytotoxicity against the human epithelioid cervical cancer cell line (HeLa).

RESULTS AND DISCUSSION

HRMS/MS Analysis of the Crude Extract. Targeted analysis of the crude extract of *N. harveyana* UHCC-0300 resulted in the discovery of 13 PUW/MINs (**1**−**13**), including 8 known and 5 unknown variants (Table 1). The fragmentation spectra obtained at a collision energy of 100 eV revealed the presence of diagnostic immonium ions $C_xH_{(2x+2)}N^+$ resulting from the cleavage of the modified FAs. This allowed us to assign FA chain lengths in the case of compounds *m/z* 1193.7143 (PUW E, **12**) and *m/z* 1191.7376 (PUW L, **13**) which possess unsubstituted C_{16} FA. Other compounds with either hydroxylated or chlorinated substitution on the FA chain generated the diagnostic ion fragment with the general molecular formula $C_xH_{2x}N^+$, which is usually the base peak of the MS/MS spectrum at high energy. This ion was followed by the product ions $C_xH_{2x+2}NO^+$ and $C_xH_{2x+2}NCl^+$ for variants bearing hydroxylated and chlorinated FAs, respectively. PUW/MIN chemical variants containing an oxo group bound to a FA chain have their diagnostic immonium ion with a general formula of $C_xH_{2x}NO^+$ (Table 1). Further, the amino acid sequences were attributed by the fragmentation spectra generated from a lower collision energy at 35−70 eV (Tables 2−4). The typical fragmentation pattern of methoxy-Thr (OMe-Thr) containing PUW is the opening of the amino acid cycle between Pro and *N*-MeAsn, followed by the loss of CH_4O from OMe-Thr. Subsequently, the consecutive losses of *N*MeAsn, Dhb (dehydrobutyrine), Gly/Ala, Gln, Val/Thr/Ser, Thr, Dhb, and Val were observed. Alteration of amino acids in the peptide ring, for example, Gly to Ala or Val to Ser to Thr, has almost no effect on the retention time (RT) of the compound (Table 1). However, substitution on the FA side chain led to significant shifts in the RT on a reversed-phase column as observed previously.[27] PUW/MIN variants containing hydroxylated FA chains were eluted first, followed by variants with oxo and chloro

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Table 1. High-Performance Liquid Chromatography−High-Resolution Mass Spectrometry (HPLC−HRMS) Data of the *N. harveyana* Strain UHCC-0300 Crude Extracts[a]

RT (min)	$[M + H]^+$	formula	error (ppm)	FA-diagnostic ion (m/z)	formula	error (ppm)	FA chain	sequence of amino acids	compounds
11.2	1209.7116	$C_{56}H_{97}N_{12}O_{17}^+$	2.2	224.2361	$C_{15}H_{30}N^+$	5.3	C_{16} (OH)	FA-Val-Dhb-Thr-**Thr**-Gln-**Gly**-methoxyThr-*N*-MeAsn-Pro	PUW H (**1**)
				242.2473	$C_{15}H_{32}NO^+$	2.2			
11.4	1223.7273	$C_{57}H_{99}N_{12}O_{17}^+$	2.2	224.2366	$C_{15}H_{30}N^+$	2.9	C_{16} (OH)	FA-Val-Dhb-Thr-**Thr**-Gln-**Ala**-methoxyThr- *N*-MeAsn-Pro	PUW I (**2**)
				242.2496	$C_{15}H_{32}NO^+$	7.2			
12.6	1237.7433	$C_{58}H_{101}N_{12}O_{17}^+$	2.5	252.2683	$C_{17}H_{34}N^+$	0.9	C_{18} (OH)	FA-Val-Dhb-Thr-**Thr**-Gln-**Gly**-methoxyThr-*N*-MeAsn-Pro	MIN K (**3**)
				270.2798	$C_{17}H_{36}NO^+$	2.4			
12.7	1221.7115	$C_{57}H_{97}N_{12}O_{17}^+$	2.1	268.2626	$C_{17}H_{34}NO^+$	3.2	C_{18} (O)	FA-Val-Dhb-Thr-**Ser**-Gln-**Gly**-methoxyThr-*N*-MeAsn-Pro	PUW J (**4**)
13.0	1235.7260	$C_{58}H_{99}N_{12}O_{17}^+$	1.2	268.2627	$C_{17}H_{34}NO^+$	2.8	C_{18} (O)	FA-Val-Dhb-Thr-**Thr**-Gln-**Gly**-methoxyThr-*N*-MeAsn-Pro	PUW A (**5**)
13.2	1249.7429	$C_{59}H_{101}N_{12}O_{17}^+$	2.2	268.2621	$C_{17}H_{34}NO^+$	5.2	C_{18} (O)	FA-Val-Dhb-Thr-**Thr**-Gln-**Ala**-methoxyThr-*N*-MeAsn-Pro	MIN E (**6**)
13.5	1233.7471	$C_{59}H_{101}N_{12}O_{16}^+$	1.4	268.2645	$C_{17}H_{34}NO^+$	3.7	C_{18} (O)	FA-Val-Dhb-Thr-**Val**-Gln-**Gly**-methoxyThr-*N*-MeAsn-Pro	MIN L/PUW B (**7**)
13.7	1247.7621	$C_{60}H_{103}N_{12}O_{16}^+$	0.9	268.2638	$C_{17}H_{34}NO^+$	1.0	C_{18} (O)	FA-Val-Dhb-Thr-**Val**-Gln-**Ala**-methoxyThr-*N*-MeAsn-Pro	MIN H (**8**)
14.2	1227.6791	$C_{56}H_{96}ClN_{12}O_{16}^+$	3.3	224.2372	$C_{15}H_{30}N^+$	0.4	C_{16} (Cl)	FA-Val-Dhb-Thr-**Thr**-Gln-**Gly**-methoxyThr-*N*-MeAsn-Pro	PUW C (**9**)
				260.2149	$C_{15}H_{31}ClN^+$	3.7			
14.4	1241.6948	$C_{57}H_{98}ClN_{12}O_{16}^+$	3.3	224.2368	$C_{15}H_{30}N^+$	1.9	C_{16} (Cl)	FA-Val-Dhb-Thr-**Thr**-Gln-**Ala**-methoxyThr-*N*-MeAsn-Pro	PUW K (**10**)
				260.2144	$C_{15}H_{31}ClN^+$	1.7			
14.8	1225.7011	$C_{57}H_{98}ClN_{12}O_{15}^+$	4.4	224.2376	$C_{15}H_{30}N^+$	1.4	C_{16} (Cl)	FA-Val-Dhb-Thr-**Val**-Gln-**Gly**-methoxyThr-*N*-MeAsn-Pro	PUW D (**11**)
				260.2121	$C_{15}H_{31}ClN^+$	7.2			
15.1	1193.7143	$C_{56}H_{97}N_{12}O_{16}^+$	0.2	226.2529	$C_{15}H_{32}N^+$	0.1	C_{16}	FA-Val-Dhb-Thr-**Thr**-Gln-**Gly**-methoxyThr-*N*-MeAsn-Pro	PUW E (**12**)
15.7	1191.7376	$C_{57}H_{99}N_{12}O_{15}^+$	2.4	226.2533	$C_{15}H_{32}N^+$	1.8	C_{16}	FA-Val-Dhb-Thr-**Val**-Gln-**Gly**-methoxyThr-*N*-MeAsn-Pro	PUW L (**13**)

[a]The RTs, protonated molecules ($[M + H]^+$) and molecular formula provided for the experimental m/z, FA-diagnostic ion (m/z), sequence of amino acids, and error in ppm for the compounds detected. Dhb = dehydrobutyrine. Variable amino acid positions (5 and 7) are presented in bold.

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Table 2. Precursor Ion (m/z) Data for Compounds 1, 3, 5, 9, and 12

fragment ion assignment	PUW H (1)	MIN K (3)	PUW A (5)	PUW C (9)	PUW E (12)
$[M + H]^+$	1209.7105	1237.7433	1235.7260	1227.6791	1193.7143
$[M-CH_4O + H]^+$	1177.68	1205.71	1203.70	1195.65	1161.70
$[M-CH_4O$-*N*-MeAsn $+ H]^+$	1049.62	1077.65	1075.64	1067.59	1033.62
$[M-CH_4O$-*N*-MeAsn-Dhb $+ H]^+$	966.58	994.61	992.60	984.55	951.61
$[M-CH_4O$-*N*-MeAsn-Dhb-Gly $+ H]^+$	909.57	937.57	935.58	927.54	893.57
$[M-CH_4O$-*N*-MeAsn-Dhb-Gly-Gln $+ H]^+$	781.51	809.55	807.52	799.47	765.50
$[M-CH_4O$-*N*-MeAsn-Dhb-Gly-Gln-Thr $+ H]^+$	680.46	708.48	706.48	698.43	664.46
$[M-CH_4O$-*N*-MeAsn-Dhb-Gly-Gln-Thr-Thr $+ H]^+$	579.42	607.45	605.43	597.38	563.42
$[M-CH_4O$-*N*-MeAsn-Dhb-Gly-Gln-Thr-Thr-Dhb $+ H]^+$	496.37	524.41	522.39	514.34	480.38
FA imm ion + CH_2O + Pro		379.33	395.33	351.30	353.32
FA imm ion + H_2O + CH_2O + Pro	369.27	397.34			
FA imm ion + CH_2O		282.28	298.27	254.25	256.26
FA imm ion + H_2O + CH_2O	272.22	300.29			
FA imm ion + H_2O	242.21	270.28			
FA imm ion + HCl				260.21	
FA imm ion	224.20	252.27	268.26	224.24	226.25

Table 3. Precursor Ion (m/z) Data for Compounds 2, 6, and 10

fragment ion assignment	PUW I (2)	MIN E (6)	PUW K (10)
$[M + H]^+$	1223.7273	1249.7429	1241.6948
$[M-CH_4O + H]^+$	1191.69	1217.71	1209.66
$[M-CH_4O$-*N*-MeAsn $+ H]^+$	1063.46	1089.65	1081.60
$[M-CH_4O$-*N*-MeAsn-Dhb $+ H]^+$	980.59	1006.62	998.57
$[M-CH_4O$-*N*-MeAsn-Dhb-Ala $+ H]^+$	909.56	935.58	927.53
$[M-CH_4O$-*N*-MeAsn-Dhb-Ala-Gln $+ H]^+$	781.50	807.53	799.48
$[M-CH_4O$-*N*-MeAsn-Dhb-Ala-Gln-Thr $+ H]^+$	680.47	706.48	698.43
$[M-CH_4O$-*N*-MeAsn-Dhb-Ala-Gln-Thr-Thr $+ H]^+$	579.41	605.43	597.38
$[M-CH_4O$-*N*-MeAsn-Dhb-Ala-Gln-Thr-Thr-Dhb $+ H]^+$	496.38	522.39	514.34
FA imm ion + CH_2O + Pro	351.30	395.33	351.30
FA imm ion + CH_2O	254.25	298.27	254.25
FA imm ion + H_2O	242.25		
FA imm ion + HCl			260.21
FA imm ion	224.24	268.26	224.24

Table 4. Precursor Ion (m/z) Data for Compounds 4, 7, 8, 11, and 13

fragment ion assignment	MinL/PUW B (7)	PUW D (11)	PUW L (13)	MIN H (8)	PUW J (4)
$[M + H]^+$	1233.7471	1225.7011	1191.7376	1247.7621	1221.7115
$[M-CH_4O + H]^+$	1201.72	1193.66	1159.71	1215.73	1189.67
$[M-CH_4O$-*N*-MeAsn $+ H]^+$	1073.66	1065.62	1031.67	1087.67	1061.62
$[M-CH_4O$-*N*-MeAsn-Dhb $+ H]^+$	990.62	982.56	948.60	1004.64	978.58
$[M-CH_4O$-*N*-MeAsn-Dhb-Gly $+ H]^+$	933.60	925.54	891.59		921.58
$[M-CH_4O$-*N*-MeAsn-Dhb-Ala $+ H]^+$				933.60	
$[M-CH_4O$-*N*-MeAsn-Dhb-Gly-Gln $+ H]^+$	805.54	797.49	763.53		793.50
$[M-CH_4O$-*N*-MeAsn-Dhb-Ala-Gln $+ H]^+$				805.54	
$[M-CH_4O$-*N*-MeAsn-Dhb-Gly-Gln-Val $+ H]^+$	706.47	698.43	664.47		
$[M-CH_4O$-*N*-MeAsn-Dhb-Gly-Gln-Ser $+ H]^+$					706.47
$[M-CH_4O$-*N*-MeAsn-Dhb-Ala-Gln-Val $+ H]^+$				706.48	
$[M-CH_4O$-*N*-MeAsn-Dhb-Gly-Gln-Val-Thr $+ H]^+$	605.43	597.38	563.42		
$[M-CH_4O$-*N*-MeAsn-Dhb-Gly-Gln-Ser-Thr $+ H]^+$					605.43
$[M-CH_4O$-*N*-MeAsn-Dhb-Ala-Gln-Val-Thr $+ H]^+$				605.43	
$[M-CH_4O$-*N*-MeAsn-Dhb-Gly-Gln-Val-Thr-Dhb $+ H]^+$	522.39	515.35	480.38		
$[M-CH_4O$-*N*-MeAsn-Dhb-Gly-Gln-Ser-Thr-Dhb $+ H]^+$					522.38
$[M-CH_4O$-*N*-MeAsn-Dhb-Ala-Gln-Val-Thr-Dhb $+ H]^+$				522.39	
FA imm ion + CH_2O + Pro	395.33	351.30	353.32	395.33	395.33
FA imm ion + CH_2O	298.27	254.25	256.26	298.27	298.27
FA imm ion + H_2O					
FA imm ion + HCl		260.21			
FA imm ion	268.26	224.24	226.25	268.26	268.26

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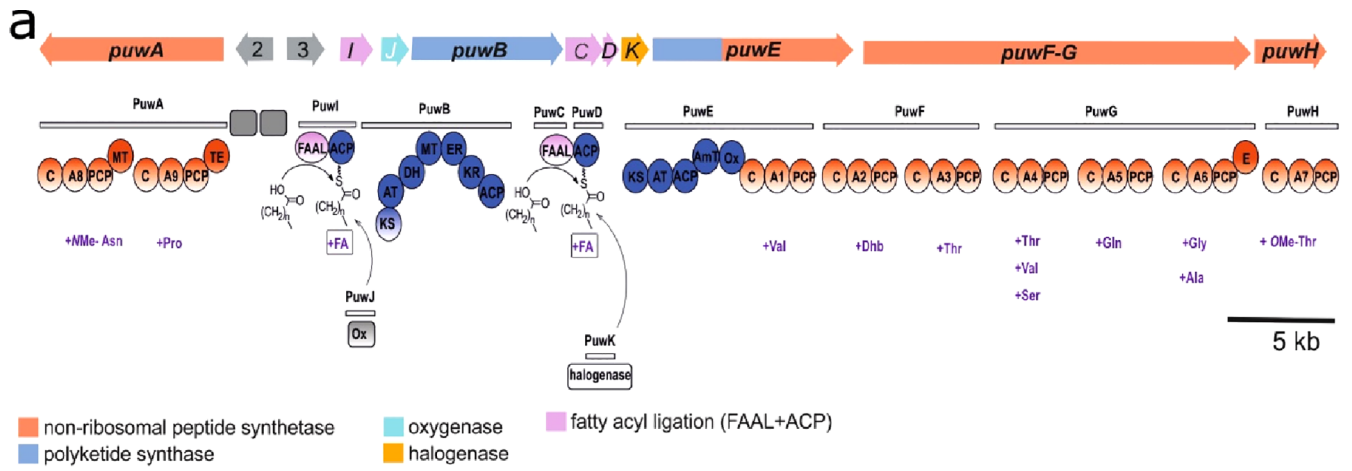

b

	PuwE A1	PuwF A2	PuwF A3	PuwG A4	PuwG A5	PuwG A6	PuwH A7	PuwA A8	PuwA A9
Cylindrospermum alatosporum CCALA 988	DAFWlGGTFK	DFWNVGMVHK	DATKVGEVGK	DFWNVGMVHK	DATKVGEVGK	DLFNNALTYK	DFWNIGMVHK	DATKVGEVGK	DVQFmAqVVK
Cylindrospermum moravicum CCALA 993	----------	----------	----------	----------	----------	----------	----------	----------	----------
Cylindrospermum alatosporum CCALA 994	----------	----------	----------	----------	----------	----------	----------	----------	----------
Anabaena sp. UHCC-0399	----I-----	----I-----	----------	----------	----------	-ILQL-A-W-	----------	----------	----I-----
Anabaena minutissima UTEX B 1613	----I-----	----I-----	----------	----------	----------	-ILQL-A-W-	----------	----------	----I-----
***Nodularia harveyana* UHCC-0300**	----I-----	----I-----	-FWN--M-H-	-AFWl-GTFK	--WQF-LID-	-ILQfvaIW-	----------	----------	----I-----
Calothrix sp. FACHB-156	----I-----	----I-----	-FWN--M-H-	-AFWl-GTFK	--WQF-LID-	-ILQfvaIW-	----------	----------	----I-----
Nostoc linckia FACHB-104	----I-----	----I-----	-FWN--M-H-	-AFWl-GTFK	--WQF-LID-	-ILQfvaIW-	----------	----------	----I-----
Trichormus sp. M33	----I-----	----I-----	-FWN--M-H-	-AFWl-GTFK	--WQF-LID-	-IvQfvaVW-	----------	----------	----I-----
Symplocastrum muelleri NIVA-CYA 644	--WW------	----I-----	-FWNI-M-H-	-A-WL-GTFK	--WQF-LID-	-ILQfGLIy-	----------	----------	----V-----

Figure 2. Structure of the puwainaphycin (*puw*) biosynthetic gene cluster organization and functional annotation of *puwA-J* genes. (**a**) Gene arrangement of the *puw* biosynthetic gene cluster in the *N. harveyana* UHCC-0300 strain and the proposed biosynthetic scheme and (**b**) comparison of A-domains of 10 *puw* biosynthetic gene clusters identified from public databases.

substitutions, and then finally variants with no substitution on the FA chain were eluted last, even though the chain lengths were two carbons less than the oxo-substituted compounds (Table 1).

Isolation and Structural Elucidation of 1−13. The benthic *N. harveyana* strain UHCC-0300 was isolated from the coastal area of the Baltic Sea in 2001[28] and grown in 100 L of Z8xS growth medium under the continuous illumination of 3.2−3.7 μmol photons m^{-2} s^{-1}.[29] The freeze-dried biomass (20 g) was extracted with 70% MeOH in water and dried under vacuum. The crude extract was subsequently fractionated using reversed-phase flash column chromatography, eluting with a mixture of H_2O/CH_3CN (from 0 to 100%) and then with 100% of MeOH, to afford 12 fractions (F1−F12). HRMS/MS analysis of all fractions indicated the presence of lipopeptides in the fractions F7−F9. Subsequent chromatography of these fractions using a Sephadex LH-20 followed by a semipreparative HPLC yielded compounds **3** (0.60 mg), **5** (9.70 mg), **6** (3.70 mg), **7** (1.20 mg), **8** (1.20 mg), **9** (0.57 mg), **10** (0.51 mg), and **11** (0.52 mg). The structures of compounds **5** and **6** were elucidated by HRMS/MS combined with nuclear magnetic resonance (NMR) data [one-dimensional (1D) and two-dimensional (2D) NMR]. The comparison of the NMR data with the data reported in the literature (Table S1) allowed the assignment of compound **5** as PUW A and compound **6** as MIN E.[18−20] Compounds **1**−**4** and **7**−**11** were identified by the HRMS fragmentation data (the amino acid sequence as well as the FA length). Data allowing fragment identification are reported in Tables 2−4. Due to the low amount of the isolated compounds, the position of substituents on the FA chain remains unresolved. Compound **12** was assigned as PUW E, with the C_{16}-FA chain. A new variant, PUW L (**13**) (FA-Val-Dhb-Thr-Val-Gln-Gly-methoxyThr-N-MeAsn-Pro), differs from PUW E (**12**) by a Val unit instead of the Thr unit. The other new variants detected were designated as PUW H (**1**), PUW I (**2**), PUW J (**4**), and PUW K (**10**). PUW H (**1**) and PUW I (**2**) bearing hydroxylated C_{16}-FA only differs from each other by a Gly/Ala unit, whereas PUW K (**10**) containing the same amino acid sequence as PUW I (**2**) differs by bearing a chlorinated C_{16}-FA. PUW J (**4**) and PUW A (**5**) bearing oxo-C_{18}-FA differs from each other by a Ser/Thr unit.

Elucidation of the Puw/Min Biosynthetic Gene Cluster in *N. harveyana* UHCC-0300. PUW/MINs were previously reported to be synthesized via a hybrid NRPS/PKS machinery encoded in 60−64 kb biosynthetic gene clusters.[24,30] In this study, we identified the 63 kb *puw* biosynthetic gene cluster using tBLASTn searches against the standalone database based on a draft genome sequence for *N. harveyana* UHCC-0300. These searches were conducted using the biosynthetic enzymes reported previously (*puw* biosynthetic gene clusters).[18,23] The *puw* biosynthetic gene cluster from the *N. harveyana* strain UHCC-0300 encoded 13 biosynthetic proteins organized in a bidirectional operon (Figure 2A). This *puw* biosynthetic gene cluster encoded two alternative loading modules, PuwI and PuwC, for the activation of different FA starter units as reported previously in the case of *Anabaena* sp. strains.[23] The FA moiety is then elongated by the PuwB and PuwE PKSs identically as in other *puw* biosynthetic gene clusters.[18,23] Biosynthetic genes encoding the PuwK accessory halogenase and the PuwJ monooxygenase were also present (Figure 2A). The presence of two alternatives PuwI and PuwC as FAAL starters was reported to result in the synthesis of lipopeptides with FA moieties of differing lengths and functionalization in *Cylindrospermum* and *Anabaena* strains.[8,23] However, in *N. harveyana* strain UHCC-0300, the FA moieties

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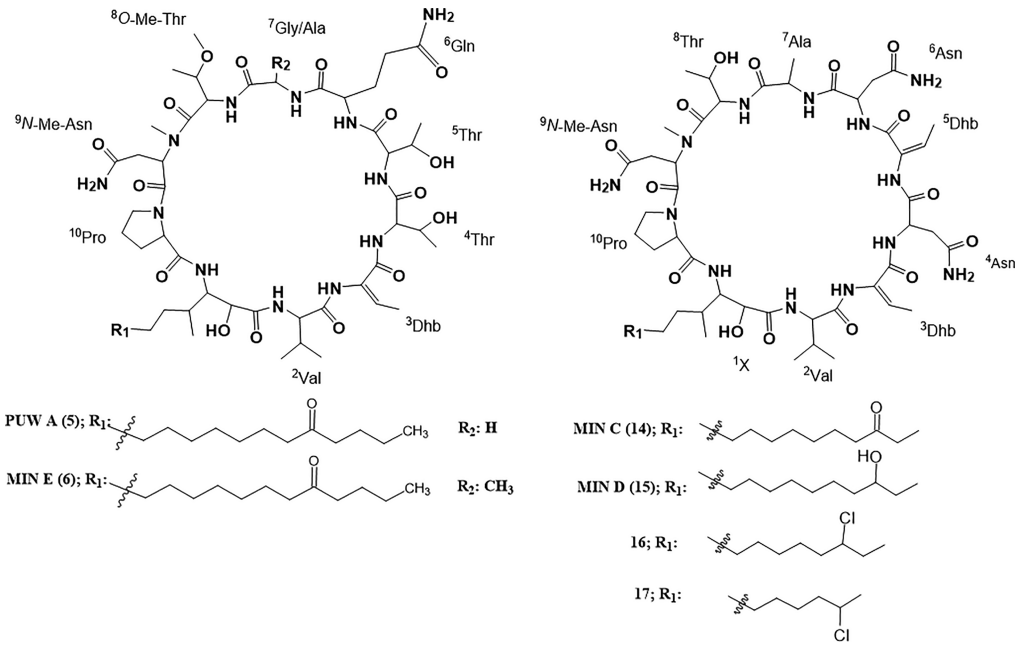

Figure 3. Structures of **5**, **6**, and **14**−**17**.

activated by the individual starter modules appear differently (Figure 2A). PuwC was previously corroborated to activate FA chains resulting in the production of variants with a shorter FA moiety (C_{10}−C_{14}) in *Cylindrospermum* and *Anabaena* strains,[24,30] while PuwI tends to activate longer FA residues resulting in the production of variants with C_{16}−C_{18} FA in *Symplocastrum* and *Anabaena* strains.[24] It is notable that in *Anabaena minutissima* UTEX B1613, which encodes both PuwC and PuwI together with the accessory halogenase PuwK and oxygenase PuwJ, the substitution pattern differs for FA with different lengths. Puw/Min variants with short FA chain (activated by PuwC) are halogenated, while the long-tail FA PUW/MIN variants (activated by PuwI) contain only hydroxyl- or oxo-substitution. Among PUW/MINs produced by *N. harveyana* UHCC-0300 (containing PuwC, PuwI, PuwK, and PuwJ), variants with only C_{16} and C_{18} FA chains were detected. While both the variants (C_{16} and C_{18}) contain hydroxyl- or oxo-substitution (compounds **1**−**8**), only the C_{16} variants were found with chlorine substitution (compounds **9**−**11**). Based on our data, we assume that the PuwC enzyme cooperates with PuwK to generate halogenated variants and PuwI/PuwJ activity results in hydroxyl-/oxo-substituted variants, which was in accordance with the previously published report. Moreover, in *N. harveyana* UHCC-0300, PuwC has apparently altered substrate specificity (activation of C_{12} FA resulting in PUW/MIN with C_{16} FA chain after elongation by PuwB/PuwE) compared to PuwC in *Cylindrospermum*/*Anabaena* reported previously (activation of C_6−C_{10} resulting in PUW/MIN with C_{10}−C_{14} FA chain after PuwB/PuwE elongation).

The PuwA, PuwE, PuwF, PuwG, and PuwH enzymes catalyze the incorporation of nine amino acids into the growing peptide chain (Figure 2). Minor variants were observed involving substitution of amino acids similar in structure and hydrophobicity, including Thr to Val (compounds **7**, **8**, **11**, and **13**) or Thr to Ser (compound **4**) at position 5 and Gly to Ala at position 7 (compounds **2**, **6**, **8**, and **10**) (Table 1). This suggests probable substrate promiscuity of the PuwG A4 and PuwG A6 adenylation domains. The stereochemistry of amino acid residues present in PUW/MIN has been widely studied. All the residues forming the peptide cycle were proved to be present in the L-form. The only exception is the presence of D-Ala at position 7, which was due to the presence of an epimerase domain in all the known Puw/Min biosynthetic gene clusters published previously. Similarly, in the proposed biosynthetic scheme, an epimerase domain was found with PuwG, suggesting the presence of D-Ala. Based on this, we concluded that the stereochemistry of the obtained PUW/MIN is identical as proved previously.[20] We conducted BLASTp searches using conserved enzymes from the *N. harveyana* UHCC-0300 *puw* biosynthetic gene cluster against the nonredundant database at NCBI and identified 10 complete *puw* biosynthetic gene clusters (Figure 2B). The 10 *puw* biosynthetic gene clusters shared a conserved gene order and encoded 12 *puw* biosynthetic enzymes (Figure 2B). This finding suggests that while rare, the *puw* biosynthetic gene clusters encoding alternative lipoinitiation mechanisms are widespread in the Nostocales.

Effect of FA Substitution on Bioactivity. The antiproliferative activity of PUW F, MIN A-D, and MIN E-L has been reported in the literature, with different experimental settings and on different human cell lines (HeLa, colon carcinoma HT-29, and melanoma cell MDA-MB 435, respectively).[19,20] Recently, the cytotoxic activity of PUW/MIN variants with respect to the length of the FA moiety was studied by our team, and it was concluded that in a certain FA length span (C_{12}−C_{14}), the cytotoxicity increases with the FA length but reduces further with FA extension.[26] We used identical setting of the experiment and performed the cytotoxicity assays on human HeLa cells. To obtain a broader picture, we included four PUW/MIN variants (**14**−**17**) previously isolated, differing in their amino acid positions 4−8 from the PUW/MIN variants isolated from *N. harveyana* strain UHCC-0300 (Figure 3). The four variants are as follows: MIN C (**14**) bearing an oxo group, MIN D with the hydroxylated FA moiety (**15**) isolated from *Anabaena* sp.

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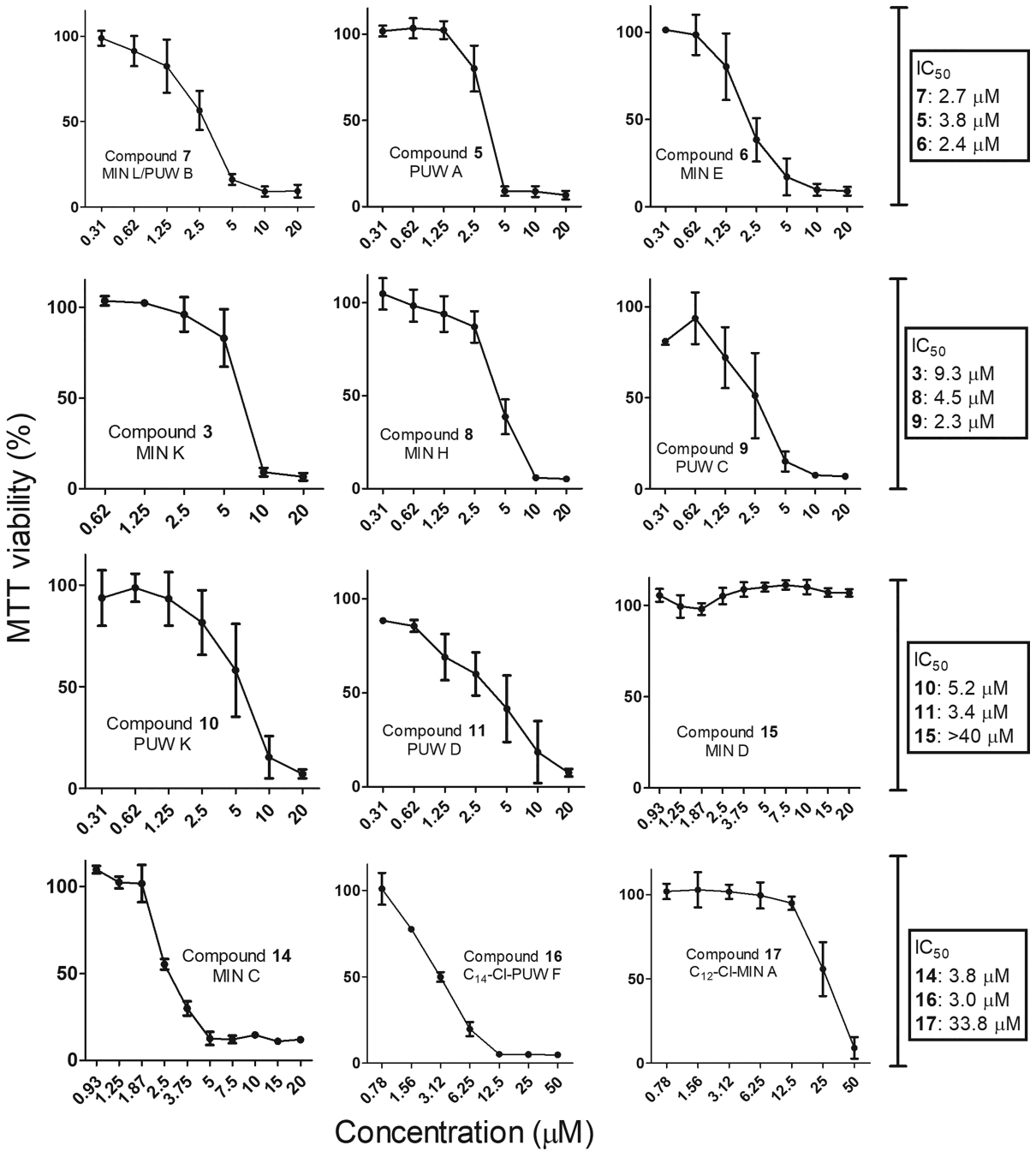

Figure 4. Cell viability was assessed by the MTT assay at a 48 h exposure time. The cell viability was calculated as the percentage of viable cells in compound-treated cells relative to control. All the experiments were performed with at least three independent biological replicates. Data from repeated measurements were shown as the mean ± SE. IC_{50} values were calculated using GraphPad Prism 5.

UHCC-0399, and the chlorinated variants PUW F possessing C_{14}−Cl FA (**16**) and MIN A possessing C_{12}−Cl FA (**17**) isolated from *Cylindrospermum alatosporum* CCALA 988[24,31] (Figure 3).

Compounds **5**−**8**, bearing the oxo substitution on the C_{18} FA chain, manifested a moderate cytotoxic effect with IC_{50} values of 3.8 ± 0.8, 2.4 ± 0.5, 2.7 ± 0.5, and 4.5 ± 0.4 μM, respectively, on HeLa cell lines. A similar IC_{50} value (3.8 ± 0.5 μM) was observed for compound **14** bearing the oxo group on the C_{16} FA chain, differing from compounds **5**−**8** in the peptide core sequence (Table 1). A lower potency (IC_{50} of 11.8 μM) was recorded for compound **14** in human colorectal HT-29 cells.[19] Concerning the hydroxylated variant, compound **3** showed a weaker potency with an IC_{50} value of 9.3 ± 3.2 μM. Interestingly, compound **15** (C_{16}, OH) was not cytotoxic up to the highest tested concentration of 40 μM, whereas previously, it was observed to possess a weak cytotoxicity on HT-29 cells (IC_{50} = 22.7 μM).[14,19] Finally, variants with chlorine substitution on C_{16} FA (compounds **9**−**11**) displayed IC_{50} values of 2.3 ± 0.9, 5.2 ± 1.4, and 3.4 ± 1.3 μM, respectively. Chlorinated compounds **16** and **17** with C_{14} FA manifested very variable IC_{50} values of 3.09 ± 0.3 and 33.89 ± 6.3 μM, respectively, which is likely the result from different positions of the Cl substituent of the FA (Figure 4). The chlorinated variants with C_{14} FA were previously shown to possess IC_{50} values of 1.2 and 2.6 μM against MDA-MB-435

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cells.[20] Although non-substituted PUW/MIN variants were not subjected to the cytotoxicity test in this study, it is worthy to mention that PUW F bearing unsubstituted C_{14} FA showed moderate IC_{50} values against HeLa with an IC_{50} value of 2.2 μM,[25] and MIN A with C_{12} FA was reported to possess a similar potency against HT-29 and HeLa cells with IC_{50} values of 2.0 μM[19] and 2.8 μM, respectively.[26]

CONCLUSIONS

The current study presents the identification of 13 PUW/MIN chemical variants produced by the *N. harveyana* strain UHCC-0300 and proposes a biosynthetic route for their production. Searches against public sequence repositories suggested the widespread occurrence of PUW/MIN biosynthetic gene clusters encoding strains in the environment. Moreover, this is the first comprehensive study reporting the anti-proliferative activity of PUW/MIN variants on human cells in vitro. Based on our findings, the following conclusions can be reported: PUW/MIN bearing unsubstituted and an oxo-substituted FA chain (C_{14}−C_{18} length) possesses moderate cytotoxicity with a comparable IC_{50} value, demonstrating that the oxo-substitution does not affect the bioactivity. In addition, our findings suggest that the differences in the PUW/MIN peptide ring at positions 4−8 have only a minimal effect on cytotoxicity. The differences between the IC_{50} values of oxo- and hydroxyl-substituted variants clearly show that the lipophilicity of the FA residue is essential for the compound's interaction with the plasma membrane.[21,25] Finally, the chlorine substitution and the position of the substitution affected largely the compound's cytotoxicity potential.

EXPERIMENTAL SECTION

Cultivation of Cyanobacterial Biomass and Crude Extract Preparation. Crude extracts were prepared following the pre-established protocol.[32,33] Briefly, freeze-dried biomass was ground with the sea sand and extracted three times with 75% MeOH in water, followed by bath sonication. The extracts were evaporated under vacuum using a rotary vacuum evaporator (Heidolph, Germany) and dissolved with 75% MeOH in water to get a final concentration of 4 mg/mL for LC−MS/MS analysis.

HPLC−MS/MS Analysis. A Thermo Scientific Dionex-UltiMate 3000 UHPLC (Thermo Scientific) equipped with a diode array detector was used for the analysis of the crude extract. HPLC separation was performed on a reversed-phase Kinetex Phenomenex C_{18} column (150 × 4.6 mm, 2.6 μm) with H_2O/CH_3CN containing 0.1% HCOOH as a mobile phase. The flow rate during analysis was 0.6 mL/min. The gradient is as follows: H_2O/CH_3CN 85/15 (0 min), 85/15 (in 1 min), 0/100 (in 20 min), 0/100 (in 25 min), and 85/15 (in 30 min). HPLC was connected to a high-resolution mass spectrometer with an electrospray ionization source (impact HD mass spectrometer, Bruker). The mass spectrometer settings are as follows: dry temperature 200 °C; drying gas flow 12 L/min; nebulizer 3 bar; capillary voltage 4500 V; and endplate offset 500 V. The spectra were collected in the range 20−2000 *m/z* with the spectra rate 4 Hz. The collision energy alternated from 35 eV or 50 to 100 eV. Calibration was determined using a LockMass 622 as an internal calibration solution and CH_3COONa clusters at the beginning of each analysis. The extract was analyzed for the characteristic β-amino FA immonium ion following the previously described protocol.[27]

Genome Sequencing, Assembling, Annotation, and Mining for the Identification of the Puw/Min Gene Cluster. *N. harveyana* UHCC-0300 was first isolated on 28/10/2001 from a plant surface at the littoral zone in Vartiokylänlahti, Helsinki, Finland. The *N. harveyana* UHCC-0300 strain was grown in a photon irradiance of 5 μmol m^{-2} s^{-1} in Z8xS medium that lacked a source of combined nitrogen. The strain was grown for 21 days at 18 °C and was harvested by centrifugation at 10,000*g* for 7 min (Sorvall LYNX 6000 Superspeed Centrifuge, Thermo Fisher Scientific). The genomic DNA of the strains was extracted from 89 mg of wet cells that were lysed with a heat-shock treatment consisting of repeated (×15) liquid nitrogen immersion and thawing at a 55 °C water bath. DNA extraction was then carried out using a commercial DNA extraction kit (E.Z.N.A. SP Plant DNA Mini Kit Protocol—Fresh/Frozen Samples, Omega Bio-Tek). The DNA yield and quality were verified by NanoDrop (NanoDrop 1000 Spectrophotometer, Thermo Fisher Scientific). The DNA size and quality were further assessed by gel electrophoresis at room temperature (100 V, 400 mA, 30 min, 0.9% agarose) in 0.5 × TAE-buffer (20 mM Tris, 10 mM acetic acid, 0.5 mM ethylenediaminetetraacetic acid, pH 8.3). Libraries were prepared with a Nextera DNA flex library prep kit (recently renamed to Illumina DNA Prep), and Illumina MiSeq sequencing was carried out using the MiSeq Reagent Kit v3 (600 cycle). The obtained sequences were trimmed to remove adapters using Cutadapt-1.9.1 with options -q 25 -m 50,[34] and the assemblies were prepared from the trimmed fastaq files using SPAdes v3.12.0 with the careful option.[35] The resulting assembly of 5.3 Mb and 113 scaffolds was then further processed for taxonomic classification using Kraken v2,[36] and contaminating scaffolds were removed with ZEUSS v1.0.2.[37]

Identification and Annotation of the *puw* Biosynthetic Gene Cluster. A 63 kb *puw* biosynthetic gene cluster was identified through tBLASTn searches using PuwA-K as query sequences against a standalone BLAST database of the *N. harveyana* UHCC-0300 genome. There was a single 127-bp gap in the *puw* biosynthetic gene cluster of *N. harveyana* UHCC-0300. This gap was closed by PCR and Sanger sequencing. The fragment containing the gap was amplified with the oligonucleotide pair puwIF (5′ TTATTCATGACTTTGGGATGATCC-3′) and gap1R (5′-TACTGGAAAATGCCCTCACCAGTTGG-3′) and then purified using the PCR clean-up kit (Macherey-Nagel NucleoSpin Gel and PCR Clean-up Kit, Fisher Scientific). Sanger sequencing of the gap was done using the primer pair gap1F (5′-GCTTTCGAGAGCGTGATTTAGGCAAAG-3′) and gap1R (5′-TACTGGAAAATGCCCTCACCAGTTGG-3′) at the Eurofins Genomics facilities. The genes encoded in the *puw* biosynthetic gene cluster were predicted using GLIMMER. Start sites were predicted, and proteins were annotated manually using a combination of searches against the Conserved Domain Database and protein classification resources at NCBI and InterProScan searches and BLASTp searches against the non-redundant database at NCBI. The annotated sequence of the *puw* gene cluster from *N. harveyana* UHCC-0300 was deposited in GenBank under the accession number OK416066. We conducted BLASTp searches using conserved enzymes from the *N. harveyana* UHCC-0300 *puw* biosynthetic gene cluster against the non-redundant database

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at NCBI in order to identify complete *puw* biosynthetic gene clusters in public databases.

Isolation and Structural Elucidation of Compounds 1−13. Large-scale cultivation (100 L) of the strain yielded 20 g of the dried biomass. The crude extract was prepared as described above. The crude extract (5 g) obtained was fractionated using reversed-phase flash column chromatography, eluting with a mixture of H_2O/CH_3CN (from 0 to 100%) and then with 100% of MeOH, to afford 12 fractions. Three fractions (F7, F8, and F9) were subjected to a Sephadex LH-20 gel chromatography column eluting with $CHCl_3$/MeOH (1:1) and subsequently purified using semipreparative reversed-phase column chromatography (Phenomenex Kinetex 5 μm EVO C18 100 Å, 200 × 10.0 mm) eluted with H_2O (A)/CH_3CN (B) both containing 0.1% HCOOH at a flow rate of 3 mL/min using the following gradient: A/B 68/32 (0 min) and 60/40 (in 50 min). The compound elution was monitored on the MWD detector set to 220 nm to obtain compounds **3** (0.6 mg), **5** (9.7 mg), **6** (3.70 mg), **7** (1.20 mg), **8** (1.02 mg), **9** (0.20 mg), **10** (0.15 mg), and **11** (0.12 mg). The NMR spectra were acquired at 25 °C on a Bruker AVANCE Neo 700 MHz spectrometer (Billerica, MA, US) equipped with a triple resonance CHN cryoprobe using DMSO-d_6 (Sigma-Aldrich, Milan, Italy) as solvents and the 1D and 2D standard pulse sequences provided by the manufacturer. The ^{1}H chemical shifts were referenced to the residual solvent's protons resonating at 2.50 (CHD_2SOCD_3) ppm. All ^{13}C-NMR chemical shifts were assigned using the 2D spectra; therefore, monodimensional ^{13}C-NMR spectra were not recorded and were referenced to the solvents' methyl carbons resonating at 39.51 ppm (DMSO-d_6). Abbreviations for signal couplings are as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and b = broad. The HR-MS/MS data (Figure S1) and the comparison of the NMR data with the data reported in the literature (Table S1) allowed the assignment of compound **5** as PUW A (Figures S2−S8) and compound **6** as MIN E[18−20] (Figures S9−S15). The HR-MS/MS fragmentation data for all the new compounds, PUW H−PUW K (**1**, **2**, **4**, **10**, and **13**), isolated are provided in Figure S16. The purity of all the other compounds proceeded further for cytotoxicity was evaluated on the basis of ^{1}H-NMR spectra (for compounds **3**, **7**, and **8**, Figures S17−S19) and UV−vis chromatogram (for compounds **8**−**11**, Figure S20).

Compound (**5**): δ 9.10 (1H, s), δ 8.75 (1H,br s), δ 8.35 (1H, d, J = 9.2), δ 7.96 (1H, t, J = 6.2), δ 7.52 (1H, s), δ 7.38 (1H, s), δ 7.30 (1H, s) δ 6.87 (1H, d, J = 8.7) δ 6.84 (1H, d, J = 10.4), δ 6.81 (2H, ovl), δ 5.97 (1H, s), δ 5.56 (1H, dd, J = 11.6,3.0), δ 5.53 (1H, d, J = 4.5), δ 5.36 (1H, q, J = 7.5), δ 5.20 (1H, d, J = 5.2), δ 4.90 (1H, dd, J = 9.3, 2.4), δ 4.72 (1H, dd, J = 9.1, 2.6), δ 4.58 (1H, m), δ 4.30 (1H, dd, J = 9.1, 6.5), δ 4.27(1H, dd, J = 8.3, 1.8), δ 4.17 (3H, ovl), δ 4.10 (2H, ovl), δ 3.99 (1H, dd, J = 16.9, 7.3), δ 3.94 (1H, ddd, J = 11.0, 10.5, 5.1), δ 3.83 (1H, t, J = 3.8) δ 3.73 (1H, m) δ 3.22 (1H, dd, J = 17.1, 6.5), δ 3.18 (3H, s), δ 3.17 (1H, m), δ 3.03 (1H, dd, J = 16.7, 11.9), δ 2.96 (3H, s), δ 2.40 (4H, ddd, J = 7.7,7.0,6.0), δ 2.17 (2H, m), δ 1.98 (4H, ovl), δ 1.82 (3H, ovl), δ 1.74 (3H, d, J = 7.4), δ 1.70 (1H, m), δ 1.61 (1H, m), δ 1.45 (4H, ovl), δ 1.25 (21H, ovl), δ 1.00 (3H, d, J = 6.1), δ 0.89 (3H, d, J = 6.9), δ 0.84 (6H, ovl), δ 0.57 (3H, d, J = 6.7)

Compound (**6**): δ 9.10 (1H, s), δ 8.84 (1H, d, J = 3.5), δ 8.39 (1H, d, J = 9.7), δ 7.59 (1H, d, J = 7.5), δ 7.51 (1H, s), δ 7.28 (1H, s), δ 7.26 (1H, d, J = 7.9) δ 6.85 (1H, d, J = 8.8) δ 6.82 (1H, s), δ 6.78 (1H, d, J = 10.1), δ 6.75 (1H, d, J = 9.3), δ 6.02 (1H, s), δ 5.57 (1H, d, J = 4.4), δ 5.53 (1H, dd, J = 11.6,3.0), δ 5.39 (1H, q, J = 7.4), δ 5.29 (1H, d, J = 4.7), δ 5.02 (1H, dd, J = 9.8, 2.2), δ 4.79 (1H, dd, J = 9.5, 1.7), δ 4.59 (1H, m), δ 4.32 (1H, dd, J = 9.2, 6.5), δ 4.26(1H, dd, J = 8.2, 1.9), δ 4.19 (4H, ovl), δ 4.09 (1H, ddd, J = 10.3, 8.9, 4.6), δ 3.94 (1H, ddd, J = 11.6, 10.8, 5.1), δ 3.91(2H, ovl), δ 3.72 (1H, m), δ 3.14 (3H, s), δ 3.12 (1H, m), δ 3.00 (1H, dd, J = 15.3, 12.0), δ 2.93 (3H, s), δ 2.38 (4H, q, J = 6.8), δ 2.15(2H, m), δ 1.98 (4H, ovl), δ 1.81 (3H, ovl), δ 1.75 (3H, d, J = 7.3), δ 1.70 (1H, m), δ 1.61 (1H, m), δ 1.44 (4H, ovl), δ 1.29 (3H, d, J = 7.4), δ 1.25 (21H, ovl), δ 0.96 (3H, d, J = 6.1), δ 0.89 (3H, d, J = 6.6), δ 0.84 (6H, ovl), δ 0.58 (3H, d, J = 6.6)

Cytotoxic Activity. Besides compounds **3** and **5**−**11** isolated from *N. harveyana* UHCC-0300, the present study has included four additional variants, MIN C (**14**) and MIN D (**15**), previously isolated from the *Anabaena* sp. strain UHCC-0399, and MIN A (**17**) and 11-chloro-4-methyl-Ahdoa-PUW F (**16**) from the strain *Cylindrospermum alatosporum* CCALA 988.[24,38] A total of 12 compounds (**3**, **5**−**11**, and **14**−**17**) were tested for cytotoxicity against the human epithelioid cervical cancer cell line (HeLa). The cells were cultivated in RPMI cultivation media supplemented with 1% antibiotic−antimycotic solution, 1% L-glutamine, and 5% fetal bovine serum. The cells were seeded in a density of 10,000 cells per well 1 day prior to the experiment. On the next day, the cultivation medium was replaced with the cultivation medium containing desired concentrations (20, 10, 5, 2.5, 1.25, 0.63, and 0.31 μM) of the tested compounds. The vehicle dimethyl sulfoxide (DMSO) concentration did not exceed 0.5%. The cell viability after the 48 h exposure to the compounds was assessed using the MTT assay as reported previously.[39] The absorbance of the compound-treated cells was measured at 590 nm (reference wavelength at 640 nm) was divided by the values obtained for the control cells and expressed in percent. All experiments were performed in biological triplicates, each including a technical triplicate of each condition. The IC_{50} values were calculated using a variable slope (four-parameter) function with Hill's slope in GraphPad Prism software.

Data Deposition. The strain *N. harveyana* has been deposited to culture collection of UHCC under the strain number UHCC-0300. The Puw/Min biosynthetic gene cluster from *N. harveyana* UHCC-0300 is available under the accession number OK416066.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c07160.

NMR data of PUW A (**5**) and MIN E (**6**) in DMSO-d_6; detection of lipopeptides in cyanobacterial strains of *N. harveyana* UHCC-0300; ^{1}H-NMR spectra of PuwA (**5**) (DMSO-d_6, 700 MHz); ^{1}H-NMR spectra of PUW A (**5**) (DMSO-d_6, 700 MHz), expansion from 0 to 5 ppm; ^{1}H-NMR spectra of PUW A (**5**) (DMSO-d_6, 700 MHz), expansion from 5 to 10 ppm; COSY spectrum of PUW A (**5**) (DMSO-d_6, 700 MHz); HSQC spectrum of PUW A (**5**) (DMSO-d_6, 700 MHz); HMBC spectrum of PUW A (**5**) (DMSO-d_6, 700 MHz); NOESY spectrum of PUW A (**5**) (DMSO-d_6, 700 MHz); ^{1}H-NMR spectra of MIN E (**6**) (DMSO-d_6, 700 MHz); ^{1}H-NMR spectra of MIN E (**6**) (DMSO-d_6, 700 MHz), expansion from 0 to 5 ppm; ^{1}H-NMR spectra of MIN E (**6**) (DMSO-d_6, 700

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MHz), expansion from 5 to 10 ppm; COSY spectrum of MIN E (**6**) (DMSO-d_6, 700 MHz); HSQC spectrum of MIN E (**6**) (DMSO-d_6, 700 MHz); HMBC spectrum of MIN E (**6**) (DMSO-d_6, 700 MHz); NOESY spectrum of MIN E (**6**) (DMSO-d_6, 700 MHz); detection of lipopeptides in cyanobacterial strains of *N. harveyana* UHCC-0300; ^{1}H-NMR spectra of MIN K (**3**) (methanol-*d*4, 700 MHz); ^{1}H-NMR spectra of MIN L/PUW B (**7**) (methanol-*d*4, 700 MHz); ^{1}H-NMR spectra of MIN H (**8**) (methanol-*d*4, 700 MHz); and UV−vis chromatogram (200−800 nm) of compounds **8**−**11** (PDF)

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Notes

The authors declare no competing financial interest.

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